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PREVALENCE OF AVIAN PARAMYXOVIRUS 1 AND AVIAN INFLUENZA VIRUS IN DOUBLE-CRESTED CORMORANTS (*PHALACROCORAX AURITUS*) IN EASTERN NORTH AMERICA

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ABSTRACT: Although it is well established that wild birds, such as cormorants, carry virulent avian paramyxovirus serotype 1 (APMV-1; causative agent of Newcastle disease) and avian influenza virus (AIV), the prevalence of these viruses among Double-crested Cormorants (*Phalacrocorax auritus*) in the Great Lakes region of North America has not been rigorously studied. We determined the prevalences of APMV-1 and AIV in Double-crested Cormorants from the interior population of eastern North America. From 2009 to 2011, oropharyngeal and cloacal swabs and serum samples were collected from 1,957 individual Double-crested Cormorants, ranging from chicks to breeding adults, on breeding colony sites in Michigan, Wisconsin, and Mississippi, USA, and Ontario, Canada, as well as on the wintering grounds of migratory populations in Mississippi, USA. Prevalence of antibodies to APMV-1 in after–hatch year birds was consistently high across all three years, ranging from 86.3% to 91.6%. Antibody prevalences in chicks were much lower: 1.7, 15.3, and 16.4% in 2009, 2010, and 2011, respectively. Virulent APMV-1 was detected in six chicks sampled in 2010 in Ontario, Canada. Only one adult was positive for AIV-specific antibodies and five individuals were positive for AIV matrix protein, but the latter were negative for H5 and H7 AIV subtypes. We provide further evidence that Double-crested Cormorants play an important role in the maintenance and circulation of APMV-1 in the wild, but are unlikely to be involved in the circulation of AIV.

Key words: Avian influenza virus, avian paramyxovirus-1, Double-crested Cormorants, Great Lakes region, Newcastle disease, *Phalacrocorax auritus*, seroprevalence.

INTRODUCTION

Newcastle disease (ND), caused by virulent avian paramyxovirus serotype 1 (APMV-1), and high-pathogenic avian influenza virus (AIV) have the highest economic impact on the global poultry industry (Nayak et al., 2009; Suarez, 2010). Highly virulent strains of both viruses can cause up to 100% mortality in poultry (Alexander, 1997; Easterday et al., 1997), whereas low-virulent forms of both can cause reduced egg production, respiratory illness, and lethargy in poultry. Therefore, it is important to understand how APMV-1 and AIV are naturally maintained in wild birds to prevent and predict spillover into poultry flocks.

Maintenance of APMV-1 is not fully understood (Hanson et al., 2005); it has been detected in over 200 bird species, but in almost all cases the birds were asymptomatic, except for Rock Pigeons (*Columba livia*), cormorants (*Phalacrocorax* spp), and psittacines (Kaleta and Baldauf, 1988). Most infections among wild birds, particularly in Anseriformes (ducks, geese, and swans), were caused by

low virulent APMV-1 viruses. On the other hand, both Double-crested Cormorants (*Phalacrocorax auritus*) and Rock Pigeons maintain virulent APMV-1 strains (otherwise known as ND virus [NDV]) within their populations and are associated with ND epizootics in North and Central America (Leighton and Heckert, 2007).

The only documented widespread mortality in wild birds attributed to ND has occurred in Double-crested Cormorant chicks (less than 16 wk of age; Heckert et al., 1996; Wobeser, 1997; Glaser et al., 1999; Kuiken, 1999), whereas adults of the same species are primarily asymptomatic (Kuiken et al., 1999). The first documented ND epizootic in Double-crested Cormorants in North America occurred in 1975 in Quebec, Canada (Cleary, 1977). More recently, outbreaks have occurred in Canada and the US during the early 1990s (Wobeser et al., 1993; Glaser et al., 1999) with more frequent outbreaks occurring in the last decade (Sleeman, 2010). Furthermore, prevalence of antibody to APMV-1 is relatively high in adults (Kuiken et al., 1998; Farley et al., 2001). However, other than Kuiken et al. (1998) in Saskatchewan, Canada, no investigators have documented prevalence of antibody to APMV-1 in the interior population of Double-crested Cormorants in North America.

The primary reservoirs for AIV are wild birds in the orders Anseriformes and Charadriiformes (shorebirds, gulls, and terns; Stallknecht, 2003; Olsen, 2006). Avian influenza virus has been detected in cormorant species worldwide (Iftimovici et al., 1980; Süss et al., 1994; Artois et al., 2002); but it is less prevalent among Suliformes compared to other aquatic avian orders, such as Anseriformes (Stallknecht and Brown, 2007). For instance, Süss et al. (1994) surveyed wild bird populations for AIV in Eastern Germany from 1977 to 1989 and isolated AIV from only 18 of 4,500 Great Cormorants (*Phalacrocorax carbo*) sampled. To date, there are no published studies documenting the prevalence of AIV in Double-crested Cormorants in North America.

The North American interior Double-crested Cormorant population breeds throughout the Canadian prairie provinces and the Great Lakes, and winters in Louisiana, Mississippi, Alabama, and Arkansas (King et al., 2010). Given the demonstrated ability of Double-crested Cormorants to transmit APMV-1 to commercial poultry flocks (Heckert et al., 1996) and the high mortality experienced by poultry infected with virulent APMV-1 (Alexander, 1997), it is important to obtain a thorough understanding of the prevalence of APMV-1 in this suspected wildlife reservoir. Likewise, given the additional risk of transmission of high-pathogenic strains of AIV to poultry, it is essential to test wild birds, including cormorants, to determine their propensity to be reservoirs for AIV. Our objective was to determine the prevalences of APMV-1 and AIV in Double-crested Cormorants of the interior population, both on breeding and wintering grounds. Prevalence was measured by determining past exposure as indicated by pathogen-specific antibodies or active infection by presence of virus. The study was conducted in conjunction with an ongoing demography study of Double-crested Cormorants in Ontario, Canada (King et al., 1998, 2000; Chastant, 2008) and culling efforts in Michigan and the southeastern United States by the US Department of Agriculture (USDA), Animal and Plant Health Inspection Service, Wildlife Services.

MATERIALS AND METHODS

Capture and sampling

Double-crested Cormorants (hereafter "cormorants") were sampled in Michigan, Mississippi, and Wisconsin, USA, and Ontario, Canada, from 2009 to 2011; sampling periods and intensity varied with location and year (Table 1). In Mississippi, we sampled both wintering and breeding cormorants (Table 1); the wintering birds were presumed (based on band recovery data) to be migratory and breed in the Great Lakes region (King et al., 2010). Birds were either captured alive and released at study sites or culled during ongoing

TABLE 1. Double-crested Cormorant (*Phalacrocorax auritus*) sampling effort by region, location, colony, dates, method of collection (culled or live-captured birds), and age class, 2009–2011, in the Great Lakes region, Northwestern Ontario, Canada, and southeastern United States. Prevalence of avian paramyxovirus serotype 1 (APMV-1) antibodies (Ab) using blocking enzyme-linked immunosorbent assay (bELISA) reported as available.

Region	Location	Colony	Coordinates	Dates sampled	No. culled	No. caught	Age class[a]	Proportion APMV-1 Ab+ by bELISA (No. tested)
Great Lakes	Central Lake Michigan	Ludington Storage Plant	43°53′N, 86°27′W	2009–2011	172	—[b]		
				01 June 2009	50	—	AHY	0.90 (50)
				11 May 2010	53	—	AHY	0.83 (29)
				27 May 2010	4	—	AHY	1.00 (4)
				23 June 2011	20	—	AHY	0.85 (20)
				06 July 2011	45	—	26 Imm; 19 Ad	0.93 (44)
	Eastern Lake Ontario	East Brothers Island	44°12′N, 76°37′W	2011	—	55		
				22 June 2011	—	55	Ch	0.28 (54)
		Pigeon Island	44°3′N, 76°32′W	2009, 2010, 2011	—	204		
				01 June 2009	—	21	Ad	0.90 (21)
				17 May 2010	—	37	Ad	0.94 (36)
				21 June 2010	—	68	Ch	0.25 (68)
				19 May 2011	—	25	Ad	0.92 (25)
				22–23 June 2011	—	53	Ch	0.20 (51)
		Snake Island	44°11′N, 76°32′W	2010	—	25		
				21 June 2010	—	25	Ch	0.24 (25)
		West Brothers Island	44°12′N, 76°38′W	2009, 2010, 2011	—	119		
				01 June 2009	—	11	Ad	1.00 (11)
				17 May 2010	—	23	Ad	0.96 (23)
				21 June 2010	—	7	Ch	0.29 (7)
				18 May 2011	—	36	Ad	0.94 (36)
				23 June 2011	—	42	Ch	0.12 (41)
	North Channel, Lake Huron	Doucet Rock	46°8′N, 82°51′W	2009, 2010, 2011	—	59		
				01 June 2009	—	4	Ad	0.50 (4)
				21 May 2010	—	13	Ad	0.85 (13)
				14 July 2010	—	5	Ch	0.00 (5)
				24 May 2011	—	16	Ad	0.81 (16)
				25 June 2011	—	21	Ch	0.00 (21)
		Fortin Rocks	46°7′N, 82°48′W	2009, 2010, 2011	—	124		
				1 June 2009	—	11	Ad	1.00 (11)
				1 July 2009	—	22	Ch	0.05 (22)
				21 May 2010	—	34	Ad	0.91 (34)
				14 July 2010	—	12	Ch	0.25 (12)
				23 May 2011	—	18	Ad	0.89 (18)
				25 June 2011	—	27	Ch	0.05 (21)
		Herbert Island	46°8′N, 83°16′W	2010	—	21		
				14 July 2010	—	21	Ch	0.05 (21)
		Magazine Island	46°10′N, 82°46′W	2009, 2010, 2011	—	119		
				01 June 2009	—	13	Ad	0.85 (13)
				01 July 2009	—	20	Ch	0.00 (20)
				21 May 2010	—	22	Ad	0.73 (22)
				14 July 2010	—	16	Ch	0.06 (16)
				23 May 2011	—	25	Ad	0.76 (25)
				25 June 2011	—	23	Ch	0.30 (23)
		Manitoulin Island	45°48′N, 82°32′W	2009, 2011	51	—		
				26 September 2009	4	—	AHY	0.50 (4)
				10–11 May 2011	47	—	41 Imm, 6 Ad	0.96 (47)

TABLE 1. Continued.

Region	Location	Colony	Coordinates	Dates sampled	No. culled	No. caught	Age class[a]	Proportion APMV-1 Ab+ by bELISA (No. tested)
		Middle Grant Island	46°8′N, 83°19′W	2009, 2010	—	48		
				01 July 2009	—	12	Ch	0.00 (12)
				14 July 2010	—	36	Ch	0.03 (36)
		Robb Rocks	46°8′N, 82°43′W	2009, 2010, 2011	—	65		
				01 June 2009	—	8	Ad	1.00 (8)
				01 July 2009	—	10	Ch	0.00 (10)
				21 May 2010	—	2	Ad	0.50 (2)
				14 July 2010	—	12	Ch	0.17 (12)
				24 May 2011	—	11	Ad	1.00 (11)
				25 June 2011	—	22	Ch	0.00 (22)
		West Island	46°6′N, 83°1′W	2009, 2010	—	25		
				01 June 2009	—	10	Ad	1.00 (10)
				14 July 2010	—	15	Ch	0.00 (15)
	Northern Lake Michigan	Bellow Island	45°6′N, 85°34′W	2010, 2011	158	—		
				25 May 2010	50	—	AHY	0.96 (50)
				07 June 2010	52	—	AHY	0.94 (52)
				13 June 2011	56	—	Ad	0.98 (55)
		Hat Island, WI	45°6′N, 87°19′W	2011	53	—		
				24 May 2011	7	—	AHY	NT[c]
				13–14 June 2011	46	—	AHY	NT
		Jack Island, WI	45°10′N, 87°15′W	2011	25	—		
				25 May 2011	20	—	AHY	NT
				14 June 2011	5	—	AHY	NT
		Pilot Island, WI	45°17′N, 86°55′W	2011	—	35		
				6 July 2011	—	35	AHY	NT
		Spider Island, WI	45°12′N, 86°58′W	2011	—	33		
				7 July 2011	—	33	AHY	NT
	Straits of Mackinac	Goose Island	45°55′N, 84°25′W	2009, 2010, 2011	155	—		
				01 August 2009	29	—	UNK	0.86 (29)
				23 July 2010	51	—	UNK	0.67 (51)
				27 May 2011	32	—	Ad	0.91 (32)
				19 July 2011	43	—	5 Imm, 38 Ad	0.93 (42)
		Green Island	45°50′N, 84°44′W	2009, 2011	42	—		
				01 August 2009	21	—	UNK	0.48 (21)
				27 May 2011	21	—	Ad	0.95 (21)
Northwestern Ontario	Lake of the Woods	Manitou Island	49°9′N, 94°19′W	2009, 2010	—	66		
				1 June 2009	—	10	Ad	0.90 (10)
				1 July 2009	—	15	Ch	0.00 (15)
				26 May 2010	—	8	Ad	1.00 (8)
				19 July 2010	—	33	Ch	0.12 (33)
		Mary's Rock	49°40′N, 93°29′W	2009, 2010	—	9		
				1 June 2009	—	7	Ad	1.00 (7)
				26 May 2010	—	2	Ad	1.00 (2)
		Island N of Lemon Is.	49°39′N, 94°30′W	2009	—	1	Ad	1.00 (1)
				1 June 2009	—	1	Ad	1.00 (1)
		Island N of Bath	49°31′N, 94°25′W	2010	—	15	Ad	1.00 (15)
				26 May 2010	—	15	Ad	1.00 (15)

TABLE 1. Continued.

Region	Location	Colony	Coordinates	Dates sampled	No. culled	No. caught	Age class[a]	Proportion APMV-1 Ab+ by bELISA (No. tested)
Southeast USA	Eastern Mississippi, Mississippi Delta[d]	Port of Columbus	33°28′N, 88°26′W	2011	37	—		
				15 February 2011	37	—	13 Imm; 19 Ad	0.97 (32)
		Swamp Roost	33°1′N, 91°4′W	2010, 2011	63	—		
				17 May 2010	12	—	Ad	0.25 (12)
				17 May 2010	12	—	Ch	0.08 (12)
				10 June 2010	6	—	Ch	0.00 (6)
				03 March 2011	51		3 Imm; 48 Ad	0.96 (50)
		Swan Lake	33°6′N, 91°0′W	2010	72	—		
				12 May 2010	4	—	AHY	0.50 (4)
				12 May 2010	11	—	Ch	0.09 (11)
				17 May 2010	7	—	AHY	0.00 (7)
				17 May 2010	32	—	Ch	0.12 (32)
				10 June 2010	7	—	AHY	0.43 (7)
				10 June 2010	11	—	Ch	0.18 (11)
		Whittington Chan N	32°56′N, 90°32′W	2011	102	—		
				23 February 2011	32	—	17 Imm; 15 Ad	0.85 (13)
				1 March 2011	40	—	18 Imm; 22 Ad	1.00 (26)
				9 March 2011	19	—	3 Imm; 16 Ad	1.00 (19)
				6 April 2011	9	—	1 Imm; 8 Ad	NT
		Wolf/Broad	32°53′N, 90°31′W	2011	6	—		
				23 February 2011	6	—	1 Imm; 5 Ad	0.33 (6)

[a] Age classes: Ch = chick (<8 wk); Imm = immature (1–3 yr); Ad = adult (>3 yr); AHY = >1 yr; HY = <1 yr; UNK = unknown age (older than chick).

[b] Dashes indicate no samples collected.

[c] NT = not tested for APMV-1 antibodies.

[d] Cormorants culled from winter roosts in Mississippi February–early April departed the area to breed in the north. Cormorants culled after April were breeding individuals.

cormorant population management efforts (Dorr et al., 2010). In addition to capturing or culling birds, dead or dying cormorants and other colonial nesting birds in the breeding colony sites were recorded. All capture and sampling methods were approved by the National Wildlife Research Center (Institutional Animal Care and Use Committee [IACUC] protocol QA-1681) and Michigan State University IACUC protocol 02/09-016-00). Capturing and banding of cormorants in Ontario was covered under King's US Geological Survey banding permit (20873); transportation of blood and tissue samples (no carcasses were exported) across the US-Canada border was authorized by USDA export/import permit 109869 to C. Bolin.

Age is an important component of the study, and we assigned birds to age classes based on plumage (Hatch and Weseloh, 1999), as well as where or when they were captured or culled. The two broad categories are hatching year (HY; first-year birds) or after HY (AHY; 1 yr or older birds). We assigned birds to these categories only if we were unable to assign them to more definitive age classes. For instance, HY birds known to be <8 wk were called chicks; individuals 8 wk to 1 yr old were classified as juveniles. After–hatching year cormorants that have brown plumage and are 1–3 yr old are referred to as immature (i.e., not of breeding age); AHY cormorants greater >3 yr old are considered adults and have dark plumage, including a crest during breeding

season. Prior to 2011 we did not consistently record plumage coloration; but, given that the timing of culls in Michigan was prior to hatching and fledging of young (i.e., mid-May), we classified all culled birds in 2009 and 2010 as AHY. In the live-captured birds in Ontario, adults were captured on their breeding colony sites closely associated with their nests; hence, they are all known adults. On the same colony sites, the chicks were captured 3–5 wk after hatching. In several instances when cormorants were culled late in the season we assigned them an unknown age category because we could not distinguish juveniles from immatures (Table 1).

Breeding adult cormorants were captured using methods described by King et al. (1998), except leghold traps were set on nests near the center of the colony site. Upon capture, adults were placed in burlap bags and transported off-site for processing and pathogen sampling. None of the adults were uniquely marked during this study; therefore, it is possible the same individuals were sampled in multiple years. Chicks were captured by hand as described by Chastant (2008) and banded in 2009 and 2010 with year-specific US Fish and Wildlife Service bands; in 2011 they were not banded. Only chicks close to fledging age (≥3 wk), as determined by primary feather length (Dunn, 1975), were processed and sampled for APMV-1 and AIV. To minimize disturbance to breeding colonies, captured cormorants were transported off their colony site to a nearby location, processed within 4 hr, and released at their original colony site. Whole blood (2–3 mL) was collected in 3-mL Vacutainer tubes from the brachial vein or the medial metatarsal vein of live cormorants using a 22-gauge (breeders) or 26-gauge (chicks) needle.

We also sampled culled cormorants after removal from the colony sites. In the USA, cormorants were culled using methods described by Dorr et al. (2010) and recommended by the Public Resources Depredation Order (United States Fish and Wildlife Service, 2003). In Ontario, birds were culled by Meeker's Aquaculture at Lake Wolsey, Manitoulin Island, Canada. Culled birds were sampled within 4–12 hr of being killed. In culled AHY cormorants, blood was collected in the following order of priority: 1) cardiac puncture, 2) jugular vein, 3) pooled blood in the thoracic cavity, and 4) pooled blood from cutting the hepatic artery in the abdominal cavity, to minimize contamination with gastrointestinal flora and digestive enzymes.

Whole blood was centrifuged in the field at 1,500 × G for 10–15 min, and blood serum was extracted, placed in a cryovial, and immediately put on ice. Serum was stored at 4 C until antibody assays were performed within 4 wk of collection. Oropharyngeal and cloacal swabs were collected from each bird and placed in 3 mL of brain-heart infusion broth (Sigma-Aldrich, Saint Louis, Missouri, USA). Swab samples were immediately stored on ice in the field, then placed on dry ice in the afternoon when sampling was finished. Swab samples were transported and stored at −80 C until real-time reverse transcriptase PCR (rRT-PCR) was performed.

Serology

We analyzed serum for the presence of AIV-specific antibodies using multispecies blocking enzyme-linked immunosorbent assay (bELISA; Idexx Laboratories, Westbrook, Maine, USA) according to the manufacturers' instructions and using the kit's positive and negative controls. Sample to negative ratios <0.50 were considered positive; those ≥0.50 were considered negative. Antibodies specific to APMV-1 were detected in 2009–2010 using bELISA kits (Svanova Biotech AB, Uppsala, Sweden) and in 2011 using bELISA (ID VET, Montpellier, France), according to the manufacturers' instructions. The bELISA kits used in 2011 were compared with previous kits using multiple positive and negative controls, and were equally reliable in detecting APMV-1 antibodies. For both tests, percentage inhibition (PI) values >40 were considered positive; PI values <30 were considered negative; those between 30% and 40% were considered undetermined and the samples were retested. The PI values were calculated as described by manufacturer.

In 2009 and 2010, a subset of samples considered positive or undetermined by the APMV-1 bELISA were verified using a hemagglutination inhibition (HI) assay developed and performed by the National Veterinary Services Laboratory (NVSL) in Ames, Iowa, USA (Pedersen, 2011). Samples with titers ≥1:8 were considered positive.

Virology

Virus assays were conducted by Diagnostic Center for Population Animal Health (DCPAH), East Lansing, Michigan, USA (APMV-1 in 2009 and AIV in 2009–2010) and by the Research Technology Support Facility Genomics Core at Michigan State University (APMV-1 in 2010–2011 and AIV in 2011). The APMV-1 and AIV RNA were purified using QIAamp Viral RNA kits (Qiagen Inc., Valencia, California, USA) per manufac-

turers' protocol. A swab sample with 1 μL undiluted APMV-1–transcribed RNA, provided by NVSL, served as a positive control. In 2009–2010, oral and cloacal swabs were tested for APMV-1 and AIV by rRT-PCR following the protocols described by Wise et al. (2004) and Spackman et al. (2003), respectively. Every study year, only the matrix protein gene primers and probes described in Wise et al. (2004) were used for the APMV-1 rRT-PCR assay. However, in 2010 and 2011, the final reaction volume was 10 μL. The cycle threshold (CT) values used to determine positive results were ≤40 and 38 for AIV and APMV-1 rRT-PCR assays, respectively. In some instances, samples were found positive for AIV matrix protein; these were further analyzed by the DCPAH using a second rRT-PCR assay to determine the viral subtype (H5 or H7) as per Spackman et al. (2003). Samples positive for APMV-1 rRT-PCR were verified by virus isolation and sequencing by the NVSL.

Data analysis

A chi-square test was used to test for differences in antibody prevalences in birds among age classes and years. Given that AHY birds in Ontario were not uniquely marked, the possibility exists that the same individual was sampled in >1 yr, violating the assumption of independence for chi-square analysis. Hence, we also separately analyzed culled AHY birds. Given the number of different sites within a region and the varying number of individuals sampled at any time, we did not analyze differences among colony sites. A Kruskal-Wallis one-way analysis of variance on ranks was used to test differences in HI titers between 2009 and 2010, the only years in which HI titer data were collected. For all analyses, alpha was set to 0.05 and analyses were performed using SigmaPlot Version 11 (Systat Software, Inc., San Jose, California, USA).

RESULTS

From 2009 to 2011 we collected samples from 1,957 individual cormorants. Most samples were collected in the Great Lakes region of the United States and Canada (n=1,588), and the remaining samples were obtained from northwest Ontario (n=91) and southeast United States (n=282; Table 1).

Serology

Prevalence of APMV-1 antibodies based on bELISA in AHY birds across all years and locations was 85.2%, ranging from 0% to 100% between colony sites (Table 1). Among regions, prevalences were 85.2, 97.7, and 82.4% in the Great Lakes, Northern Ontario, and Mississippi, respectively. In the Great Lakes region, where our effort was greatest, antibody prevalences were 90.1, 90.5, and 91.5% in 2009, 2010, and 2011, respectively. The association between year and prevalence was not significant (χ^2=0.32, df=2, P=0.85). Likewise, when we analyzed only culled birds in the Great Lakes region, prevalence did not differ among years (χ^2=2.00, df=2, P=0.37). Additionally, antibody titers by HI in AHY did not differ between 2009 (n=144) and 2010 (n=273; H<0.001, P=0.993; Table 2).

In chicks, antibody prevalence among years was much lower than in AHY (χ^2=138.8, 272.8, and 316.3 for 2009, 2010, and 2011, respectively; df=1 and P<0.001 for all), and overall was 11.9% for the 3 yr combined. Antibody prevalence ranged from 0% to 30% between colony sites (Table 1) and region-wide antibody prevalence was 12.5, 8.2, and 10.8% in the Great Lakes, Northern Ontario, and Mississippi, respectively. There was an association between year and antibody prevalence among chicks in the Great Lakes region (χ^2=9.53, df=2, P=0.009). Antibody prevalence was 1.7% in 2009 and increased to 15.3 and 16.4% in 2010 and 2011, respectively.

Using a subset of samples (n=524) positive for APMV-1 antibody by bELISA, only 70.8% (n=371) were considered positive by HI (titer >1:8; Table 2). The ranges of PI values for HI-positive and HI-negative samples were 32.0–98.1 and 30.5–97.4, respectively.

In 2009 and 2011, no cormorants sampled had detectable AIV antibodies. However, in 2010, one breeding adult caught live on Magazine Rock in the North Channel of Lake Huron in Ontario was antibody positive for AIV. The bird was also antibody positive for APMV-1,

TABLE 2. Hemagglutination inhibition (HI) testing using a subset of avian paramyxovirus serotype 1 (APMV-1) antibody (Ab) positive samples, as determined by blocking enzyme-linked immunosorbent assay (bELISA), collected from Double-crested Cormorants (*Phalacrocorax auritus*) during 2009 and 2010. Titers less than 1:8 are considered nonprotective and thus negative. Order of age classes in the table also reflect the timing of sampling with after–hatching year (AHY; >1 yr) classed birds being culled early (prebreeding) in season and unknown-age (UNK; older than chick) individuals being culled late (postbreeding) in season.

Year	Age	bELISA Ab+[a]	Samples tested [b]	Titer by HI						%+ by HI[d]	Mean titer (SD)[e]
				<1:8[c]	1:8	1:16	1:32	1:64	1:128		
2009	AHY	53	46	25	13	6	2	—	—	45.6	12.03 (0.56)
	Breeder	66	66	22	25	13	6	—	—	66.7	11.86 (0.59)
	Chick	0	—	—	—	—	—	—	—	0.0	NA
	UNK	37	37	16	8	9	3	1	—	20.0	14.49 (0.72)
2010	AHY	126	120	56	42	13	8	1	—	53.3	11.31 (0.65)
	Breeder	114	114	31	42	26	11	3	1	71.9	13.11 (0.81)
	Chick	33	33	22	6	3	2	—	—	50.0	12.44 (0.65)
	UNK	34	30	12	8	4	4	1	—	56.7	14.75 (0.87)

[a] Number of samples antibody positive by bELISA.

[b] Number of samples antibody positive by bELISA that were tested by HI; problems with autoagglutination prevented the testing some samples.

[c] Samples considered negative by HI.

[d] Percentage congruency between bELISA and HI results (i.e., percentage positive by HI, of those that were antibody positive by bELISA and, were successfully tested by HI).

[e] Geometric mean (geometric standard deviation) antibody titer of the individuals positive by HI.

but negative for viral RNA for both AIV and APMV-1.

Virology

Active APMV-1 virus was detected only in 2010; nine chicks tested positive for APMV-1 by rRT-PCR, eight from southern Ontario near eastern Lake Ontario and one from the North Channel of Lake Huron. Cycle threshold values ranged from 30.7 to 34.5 (Table 3). Of these, six were characterized as virulent APMV-1 by amino acid sequencing at the fusion protein cleavage site, and virus isolation of the remaining three samples was unsuccessful. Three of the APMV-1 RNA–positive birds from eastern Lake Ontario exhibited ataxia, paresis, and torticollis, all classic clinical signs of virulent APMV-1 infection. These APMV-1–positive chicks in 2010 coincided with the only mortality or morbidity observed over the 3-yr study period.

In 2009 and 2010 all cormorants were negative for the AIV matrix protein by rRT-PCR. However, in 2011 five birds were positive for AIV matrix protein, with CT values ranging from 28.0 to 37.0. These five samples were then tested for H5 or H7 subtypes, but none were positive for either.

DISCUSSION

Our results support those of previous investigators (Heckert et al., 1996; Glaser et al., 1999) suggesting that cormorants are frequently exposed to APMV-1 and likely play a role as reservoirs for virulent APMV-1 in the wild. Conversely, we found no evidence that cormorants are a reservoir for AIV, which is consistent with the low AIV antibody prevalence observed in great cormorants in Europe, particularly for subtypes H5 and H7, which are the only highly pathogenic AIVs known to be pathogenic to poultry (Iftimovici et al., 1980; Süss et al., 1994; Artois et al., 2002). Hence, the remainder of this discussion will focus on APMV-1 in cormorants of the interior population of North America.

TABLE 3. Virulent avian paramyxovirus-1 infection in three Double-crested Cormorant (*Phalacrocorax auritus*) chicks sampled during the 2010 breeding season in the Great Lakes region. The range of cycle threshold (CT) values is reported from rRT-PCR. Titer results are based on hemagglutination inhibition (HI) assays. Clinical signs of ataxia, paresis, and torticollis were observed in three birds on Snake Island, Eastern Lake Ontario.

Location	Colony	Age	Sample Julian day	No. tested	Range of CT values	Titer by HI
Eastern Lake Ontario	Pigeon Island	Chick	172	68	31.4–34.0	<1:8–1:32
	Snake Island	Chick	172	25	30.7–34.5	0[a]–1:16
North Channel, Lake Huron	Magazine Island	Chick	195	16	30.8	0[a]

[a] Negative by blocking enzyme-linked immunosorbent assay.

During each year of the study, AHY birds of the interior cormorant population in North America exhibited consistently high prevalence of antibody to APMV-1 across their range. However, we detected no APMV-1 virus–positive AHY cormorants. Antibody prevalences in chicks varied with exceptionally low detection in 2009, followed by higher prevalences in 2010 and 2011. Perhaps coincidentally, the increase in antibody prevalence in chicks was first observed in 2010, when there was a documented ND outbreak among chicks in Ontario, Canada. Other documented cases of ND in cormorants were reported in July–October 2010 around the United States, including Maryland, Minnesota, North Dakota, and Wisconsin (United States Geological Survey [USGS], 2012; Diel et al., 2012). In 2011 there were only a few APMV-1 cases confirmed in Wisconsin (USGS, 2012). Hence, our low antibody prevalence among chicks in 2009 may reflect the lack of circulating APMV-1 that year. However, we caution that in many cases, mortality events go unreported, carcasses go untested, or cases are lumped with ones from other disease outbreaks.

Despite over two decades of APMV-1 epizootics in cormorant colonies in North America (Kuiken, 1999), many aspects of ND remain unknown, including the epidemiology of APMV-1. Historically, ND outbreaks have occurred irregularly, with two prominent outbreaks occurring in 1990 and 1992, the latter of which included the interior population of cormorants. More recently, epizootics of ND have occurred every other year on a regular basis (Alexander, 2009; Sleeman, 2010). APMV-1 maintenance and periodic outbreaks may be a function of 1) age and immune status of individuals in the population, 2) continuous transmission throughout the year, or 3) virus persistence in the environment.

Similarly to other investigators, we found that cormorant chicks (<16 wk old) are susceptible to virulent APMV-1 (Leighton and Heckert, 2007) but found no evidence of cormorants older than 16 wk being impacted or even actively infected. In the sporadic epizootics of APMV-1 in the Canadian provinces, the Upper Midwest, Nevada, and California, USA, and throughout the Great Lakes region, chicks experienced high mortality (up to 92%) from NDV (McFerran and McCracken, 1988; Wobeser et al., 1993; Meteyer et al., 1997; Kuiken et al., 1998; Glaser et al., 1999; Kuiken, 1999; Sleeman, 2010). Likewise, the anecdotal observations of mortality we observed in 2010 were chicks. Given the high reproductive success of cormorants in the Great Lakes, a high proportion of susceptible individuals enter the population each year. This process may prevent any long-lasting herd immunity and likely drive the observed biennial outbreaks of APMV-1 (van Boven et al., 2008).

Periodic outbreaks of APMV-1 may be due to continuous transmission among

cormorant populations throughout the year. However, to find support for this hypothesis we would have had to obtain virus-positive samples from cormorants throughout their annual cycle, which did not occur. Nevertheless, we do not have sufficient evidence to reject the continuous transmission hypothesis because we did not collect enough samples (<300 individuals) from the same cohort and location, per sampling period, to have 95% confidence of detecting at least 1% prevalence of active infection in the population (Hoye et al., 2010). More intensive sampling is needed in each region to be able to draw inferences about virulent APMV-1 prevalence in AHY cormorants. We did detect APMV-1 antibody–positive chicks on the breeding grounds in MS, which suggests that APMV-1 is actively circulating in the southeastern US.

The high antibody prevalence in AHY cormorants may suggest a barrier to continued transmission of APMV-1, through herd immunity. However, the lack of annual fluctuation in antibody prevalence (except that observed in chicks) may suggest otherwise. In addition, chickens that are seropositive for APMV-1 and exhibit high HI titers (1:80 to 1:160) can still have circulating virus and excrete virus from mucosal membranes (Holmes, 1979; Stone et al., 1980). Likewise, free-ranging, closed flocks of village chickens in Southeast Asia, where half of the flock possessed protective HI antibodies to APMV-1, showed that the virus persisted in the closed flock for at least 2 yr (Samuel and Spradbrow, 1989). Hence it is quite possible that antibody-positive cormorants could still be infected with and excreting live virus. In fact, two of the chicks found positive for APMV-1 by rRT-PCR were also antibody positive, with hemagglutination titers of 1:16 and 1:32. Additional studies are needed to determine the persistence of APMV-1 antibodies (but see Beard and Hanson, 1984; Kuiken, 1998) and the protective nature of those antibodies in free-living cormorants.

It is possible that APMV-1 is maintained in the environment. The virus persists in the environment at a wide range of temperatures, mediums, and pH (Leighton and Heckert, 2007). Under experimental conditions, the virus may remain viable in soil for 66 days at 20–30 C or for as long as 235 days at 3–6 C (Olesiuk, 1951). Likewise, it can remain infectious in feces and feather down for up to 535 days at low temperatures (Olesiuk, 1951). Clearly, this would suggest that the environment in the Great Lakes region is more conducive to maintaining APMV-1 than the southeastern United States. The virus is also viable across a wide range of pH (Moses et al., 1947) and direct sunlight (Skinner and Bradish, 1954). Cormorants in our study may have become infected with APMV-1 through environmental transmission; however, the probability of this being a primary means of transmission is relatively low because survival time and infectiousness of APMV-1 in field conditions is likely much lower than in experimental studies. Also, if the virus were maintained solely by the environment, we would have expected yearly outbreaks rather than the observed biennial outbreaks.

An interesting finding in our study was the difference in the bELISA and HI assay results from the same samples. Although HI assays have been, and are still, considered to be the gold standard in APMV-1 antibody detection, bELISAs for APMV-1 antibody detection in serum serve as an economical, high-throughput alternative for quickly determining seroprevalence. Previous studies have observed a positive relationship between the ability of ELISA and HI assays to detect APMV-1–specific antibodies in both commercial poultry flocks and laboratory animals (Snyder et al., 1983; Brown et al., 1990; Bell et al., 1991). Snyder et al. (1983) found that titers based on a standard serial dilution ELISA were strongly correlated with HI titers for the same sample (r=0.927). In that study, the ELISA was several times more likely to detect antibodies than HI

assays. In our study, approximately 30% of the samples that were positive by ELISA did not have detectable antibody with HI. However, without the infection history of the birds in this study we cannot rule out the possibility that the bELISA-positive samples were false positives. Without knowledge of antibody persistence in free-living cormorants, inoculation route, and dose, it is difficult to assess the inconsistent results between bELISA and HI; therefore, we recommend caution when interpreting results of bELISA to assess level of protective antibodies in cormorants.

Whether cormorants maintain APMV-1 is a question of critical importance, because they may introduce the pathogen into poultry operations, where the impact of an outbreak would be important. Cormorants' ability to transmit APMV-1 to poultry was demonstrated during the 1992 epizootic, where the same APMV-1 strain infecting cormorants was transmitted to and isolated from free-range turkey (*Meleagris gallopavo*) flocks in North Dakota, USA (Heckert et al., 1996). Furthermore, there is evidence from our study and as others (Allison et al., 2005) that APMV-1 exists on wintering grounds, and locations of roosting cormorants are known to occur near broiler operations in Mississippi (Scherr et al., 2010). The southeastern states currently have the highest concentration of poultry production in the US (USDA, 2012); hence, a spillover event to poultry would be economically damaging.

The high APMV-1 antibody prevalences observed among AHY and breeding cormorants further implicates cormorants as reservoir hosts for APMV-1; however, it remains unclear how APMV-1 is maintained year after year. Interestingly, although we detected active APMV-1 in cormorant chicks, we never detected an active APMV-1 infection in cormorants older than 5 wk, which leaves us with the question: where is the virus being maintained? A dedicated ongoing surveillance program for APMV-1 in cormorants on breeding and wintering grounds as well as migratory routes is necessary to better understand the maintenance and circulation of APMV-1 in the wild.

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